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ACTA BIOLOGICA CRACOVIENSIA Series Botanica 52/2: 50–58, 2010
DOI: 10.2478/v10182-010-0023-7

MICROPROPAGATION OF *OCIMUM KILIMANDSCHARICUM* GUERKE (LABIATAE)

SOUMEN SAHA, TULSI DEY, AND PARTHADEB GHOSH*

Cytogenetics and Plant Biotechnology Research Unit, Department of Botany, University of Kalyani, Kalyani-741235, West Bengal, India

Received March 13, 2010; revision accepted November 3, 2010

An efficient plant regeneration protocol has been developed from nodal explants of *Ocimum kilimandscharicum* Guerke, a medicinally important herbaceous plant species belonging to the family Lamiaceae. Axillary shoot bud proliferation was initiated from nodal explants cultured on MS medium supplemented with various concentrations of 6-benzyladenine (BA) (0.5–3.0 mg/l), kinetin (KN) (0.5–3.0 mg/l) and 2-isoPentenyladenine (2-iP) (0.5–3.0 mg/l). The maximum number of shoots (6.09±0.05), with average length 3.83±0.11 cm, was achieved with medium containing 1.0 mg/l BA. Shoot culture was established by repeated subculturing of the original nodal explants on shoot multiplication medium after each harvest of newly formed shoots. In this way, 20–30 shoots were obtained from a single nodal explant after 5 months. Rooting of shoots was achieved on half-strength MS medium supplemented with 1.5 mg/l Indole-3-butyric acid (IBA) and 2% sucrose. Well-developed plantlets transferred to plastic pots containing soil and vermiculite (1:1) showed 81.13% survival. The genetic fidelity of in-vitro-raised field-grown plants to the donor plant was ascertained from random amplified polymorphic DNA (RAPD) markers. This protocol can be used for commercial propagation and for future genetic improvement studies.

Key words: *Ocimum kilimandscharicum*, nodal segments, in vitro propagation, genetic fidelity.

INTRODUCTION

Medicinal plants are an important source of compounds for the pharmaceutical industry and traditional medicine. About 80% of the population living in developing countries still use traditional medicines derived from plants for their primary health care needs (Cunningham, 1993; De Silva, 1997). The success of any health care system depends on the availability of suitable drugs on a sustainable basis. Although synthetic drugs and antibiotics are essential for current medical practice, plants provide a major contribution to the pharmaceutical industry (Fowler, 1983; Sahoo et al., 1997). Unfortunately, rapid industrialization and urbanization has led to overexploitation and loss of valuable natural resources, including medicinally important herbaceous plants. Many species are subject to extensive, unregulated collection and are endangered or threatened with extinction (Arora and Bhojwani, 1989; Purohit et al., 1994; Sudha and Seeni, 1994).

Ocimum kilimandscharicum is an economically important medicinal perennial herb. It belongs to the family Lamiacea and is distributed in East Africa, India and Thailand (Kokwaro, 1976; Paton, 1991). It is extensively grown in the Tropics (Demissew and Asfaw, 1994). In traditional medicine, this plant is widely used for the treatment of various ailments including colds, coughs, abdominal pains, measles and diarrhea (Obeng-Ofori et al., 1998). It is also considered a source of aromatic compounds and essential oils containing biologically active constituents that act as insect repellents, particularly against mosquitoes and storage pests (Hassanali et al., 1990; Jembere et al., 1995; Bekele et al., 1996; Obeng-Ofori and Reichmuth, 1997; Kweka et al., 2008) or show antibacterial (Prasad et al., 1986) and antioxidant activity (Hakkim et al., 2008). Ethnobotanical surveys report the traditional use of basil, especially camphor-containing basil, as a veterinary medicinal as well (Baerts and Lehmann, 1991). The seed oil of *Ocimum kilimandscharicum* contains camphor as the most active component (Jembere et al., 1994); this oil is suitable for industrial purposes (Angers et al., 1996). It is also used in the Mediterranean area in interesting forms for decorative purposes (Simon and Reiss-Bubenheim, 1987; Morales et al., 1993b).

e-mail: pdgbot@rediffmail.com

PL ISSN 0001-5296

The major difficulty in the use of Lamiaceae species for pharmaceutical purposes is its individual variability, due to genetic and biochemical heterogenicity (Shetty, 1997; Viera et al., 2001; Dode et al., 2003). The conventional method of propagating this species is through seeds, but seed viability is very poor and low germination limits its multiplication. Moreover, seed-derived progenies are not true to type, due to cross-pollination (Heywood, 1978).

In vitro culture techniques offer an option for the study and conservation of rare, threatened or endangered medicinal plants (Mauseth, 1979; Ajithkumar and Seeni, 1998; Sahoo and Chand, 1998; Prakash et al., 1999), and a tool for efficient and rapid multiplication of species when high uniformity of progeny is required. There is increasing interest in using these techniques for rapid and large-scale propagation of medicinal and aromatic plants (Bajaj et al., 1988; Vincent et al., 1992; Krishnan and Seeni, 1994; Purohit et al., 1994; Sudha and Seeni, 1994; Bhat et al., 1995; Patnaik and Debata, 1996; Sahoo et al., 1997). Many in vitro studies have been conducted on Lamiaceae species, including *Ocimum basilicum* L. (Ahuja et al., 1982; Sahoo et al., 1997; Begum et al., 2002; Saha et al., 2010), *Ocimum gratissimum* (Gopi et al., 2006) and *Ocimum sanctum* (Singh et al., 1999; Begum et al., 2000; Banu et al., 2007), but to our knowledge there are no reports on in vitro propagation of *Ocimum kilimandscharicum* through nodal explants. In this work we established a reliable plantlet regeneration protocol using nodal explants for large-scale production of *Ocimum kilimandscharicum* and for long term germplasm storage in vitro for conservation. We also assessed the genetic fidelity of the micropropagated *O. kilimandscharicum* plants. The protocols presented here should become a valuable part of future efforts at genetic improvement of this important medicinal plant.

MATERIAL AND METHODS

PLANT MATERIALS

Young nodal explants (1–2 cm) were collected from 2–3-month-old plants of *Ocimum kilimandascharicum* maintained in the medicinal and aromatic plant garden of the Department of Botany, University of Kalyani (Kalyani, India), washed thoroughly under running tap water and then treated with 5% (m/v) Teepol (Qualigen, Mumbai, India) for 15–20 min, followed by 3–5 rinses in sterile distilled water. The nodal segments were then surface-sterilized with 70% alcohol for 1 min followed by immersion in 0.1% (m/v) aqueous mercuric chloride ($HgCl_2$) solution for 3–5 min and finally rinsed 4 or 5 times with autoclaved sterile distilled water in a flow chamber. The surface-sterilized explants were trimmed at the cut ends to 1–1.2 cm prior to inoculation on culture media.

CULTURE MEDIA AND CONDITIONS

Initially the shoot tips were cultured on MS basal medium containing 3% (m/v) sucrose (Himedia, Mumbai, India) for culture initiation. They served as explant sources for subsequent experiments. The pH of the medium (supplemented with the respective growth regulators) was adjusted to 5.7 with 1 N NaOH or 1 N HCl before gelling with 0.8% (m/v) agar (Himedia, Mumbai, India). All the experiments used analytical grade chemicals. The explants initially were implanted vertically on the culture medium in test tubes (150 × 25 nm) and plugged tightly with nonabsorbent cotton. All the cultures were kept under cool-white fluorescent light (16 h photoperiod, 40 μmol m^{-2} s^{-1}, Philips, India) at 25±2°C and 60–70% relative humidity.

SHOOT PROLIFERATION

For axiliary shoot bud proliferation, the nodal explants were placed on MS medium supplemented with various concentrations of 6-benzyladenine (BA, 0.5–3.0 mg/l) or kinetin (KN, 0.5–3.0 mg/l) or 2-iso Pentenyladenine (2-iP, 0.5–3.0 mg/l) as sole growth regulator. Each treatment was done three times in 10–12 replicates. Data were recorded after 4 weeks of culture.

SHOOT MULTIPLICATION

When these axillary shoots attained length of 3–4 cm they were excised, cut into single nodal segments and further multiplied for four or five subcultures at 3-week intervals on MS media containing 1.0 mg/l BA.

IN VITRO ROOTING

For root induction, shoot tips with 3 or 4 fully expanded leaves from plants grown in vitro were cultured on half- or full-strength MS basal medium supplemented with different concentrations of α-Napthaleneacetic acid (NAA) (0.5–2.0 mg/l) or IBA (0.5–2.0 mg/l). The number of shoots that produced roots was recorded after three weeks of incubation.

ACCLIMATIZATION OF REGENERATED PLANTS

Complete rooted plantlets with 7–10 fully expanded leaves were removed from the culture medium and the roots were washed gently under running tap water to remove agar. The plantlets were trans-

ferred to plastic pots (5 cm diam.) containing a mixture of sterilized garden soil and vermiculite (1:1) and covered with transparent plastic bags to ensure high humidity. The growth chamber was maintained at 26±1°C, 80–85% relative humidity and light intensity 50 µmol m^{-2} s^{-2} under a 16 h photoperiod in culture room conditions; the chamber was opened gradually during an acclimatization period of 3 weeks. After acclimatization the plantlets were transferred to a greenhouse with simulated habitat and the survival percentages were recorded.

DNA ISOLATION AND PCR AMPLIFICATION

Genomic DNA was extracted from young leaves of in-vitro-raised field-grown plants of *Ocimum kilimandscharicum* and the mother plant by the Cytl trimethyl ammonium bromide (CTAB) procedure (Murray and Thompson, 1980) with some modification of the extraction buffer. DNA purity was checked on 0.8% agarose gel and also from values of the 260/280 nm UV absorbance ratio (Sambrook et al., 2001). For the polymerase chain reaction (PCR) for DNA amplification we used ten arbitrary decamer RAPD primers (Bengalore Genni Pvt. Ltd., India) with the following sequences: MS10C1: GCACGCCGGA, MS10C2: AAATCGGAGC, MS10C3: GTCCTACTCG, MS10C4: GTCCTTAGCG, MS10C5: TGCGCGATCG, MS10C6: AACGTACGCG, MS10C7: CTATCGCCGC, MS10C8: CTCTCCGCC, MS10C9: GGATGAGACC, MS10C10: AGGGCCGTCT. DNA fingerprinting profiles were compared to evaluate clonal fidelity and genetic stability. Amplification was performed in 25 µL PCR reaction mixture consisting of 2.5 µL Taq buffer, 1 µL dNTPs, 0.5 µL Taq polymerase, 1 µL DNA (approximate 25 ng/µL), 1.0 µL primer (10 pmol), 1.5 µL $MgCl_2$ and 16.5 µL water. The PCR reaction steps were as follows: preheating for 5 min at 94°C; 40 cycles of 1 min at 94°C, 2 min at 35°C and 2 min at 72°C, and elongation completed by final extension of 5 min at 72°C. After amplification, the PCR product was resolved by electrophoresis in 1.4% agarose gel (Himedia, Mumbai, India) and stained with ethidium bromide (0.5 µg/ml). 100–3000bp λ DNA digested with *EcoRI* and *Hind*III was used as the DNA marker, and bands were visualized under UV light and photographed using Gel Doc equipment (Bio Rad). The PCR reaction was performed at least twice to check reproducibility.

STATISTICAL ANALYSIS

The experiments followed a completely randomized design and were done at least three times. Ten to twelve explants per replicate were used in each treatment. Data were analyzed by one way ANOVA and the mean values from treatments were compared using Tukey's HSD test at $p = 0.05$ with SPSS ver. 10. The results are expressed as means ±SE of three experiments.

RESULTS AND DISCUSSION

SHOOT INDUCTION

Ocimum kilimandscharicum plants were efficiently regenerated from nodal explants. When nodal explants from field-grown mature plants of *Ocimum kilimandscharicum* were cultured on MS medium supplemented with various concentrations of BA, KN and 2-iP, the emergence of the adventitious shoot buds was observed at 12–15 days after inoculation (Fig. 1a). Data on different growth parameters from different treatments were recorded 4 weeks after culture initiation following one transfer to new medium (Tab. 1). The cytokinin type and concentration greatly influenced axillary shoot regeneration from nodal explants. Medium without growth regulator (control) gave no regeneration response; the explants swelled and became necrotic 2 weeks after inoculation. Of the three cytokinins tested, BA-treated explants achieved higher regeneration than those treated with KN and 2-iP. The 1.0 mg/l BA treatment yielded maximum regeneration (93.88%) and maximum number of multiple shoots (6.09±0.05). At 4 weeks, shoots developed in this medium also were longest (3.83±0.11 cm). At BA concentrations higher than 1.0 mg/l, the number of shoots as well as the percentage response were lower (Tab. 1). Reduction of the number of shoots generated from each node at higher than optimal BA concentrations have also been reported in several medicinal plants (Kukreja et al., 1990; Sen and Sharma, 1991; Vincent et al., 1992). Of the three cytokinins tested (BA, KN, 2-iP), BA was most effective in inducing multiple shoot formation. In other work, BA has been shown to overcome apical dominance, release lateral buds from dormancy and promote shoot formation (George, 1993). Stimulation of multiple shoot formation by BA has been reported in several medicinal plant species including *Ocimum basilicum* (Ahuja et al., 1982; Sahoo et al., 1997; Begum et al., 2002; Saha et al., 2010), *Curcuma* and *Zingiber officinale* (Balachandran et al., 1990), *Piper* spp. (Bhat et al., 1995), *Tylophora indica* (Faisal et al., 2007) and *Mentha piperita* (Saha et al., 2010).

NODAL MULTIPLICATION OF SHOOT CULTURES

Shoot cultures were multiplied by repeatedly subculturing the original nodal explants on shoot multiplication medium (MS supplemented with 1.0 mg/l BA) after 4 weeks of culture. Shoot generation abili-

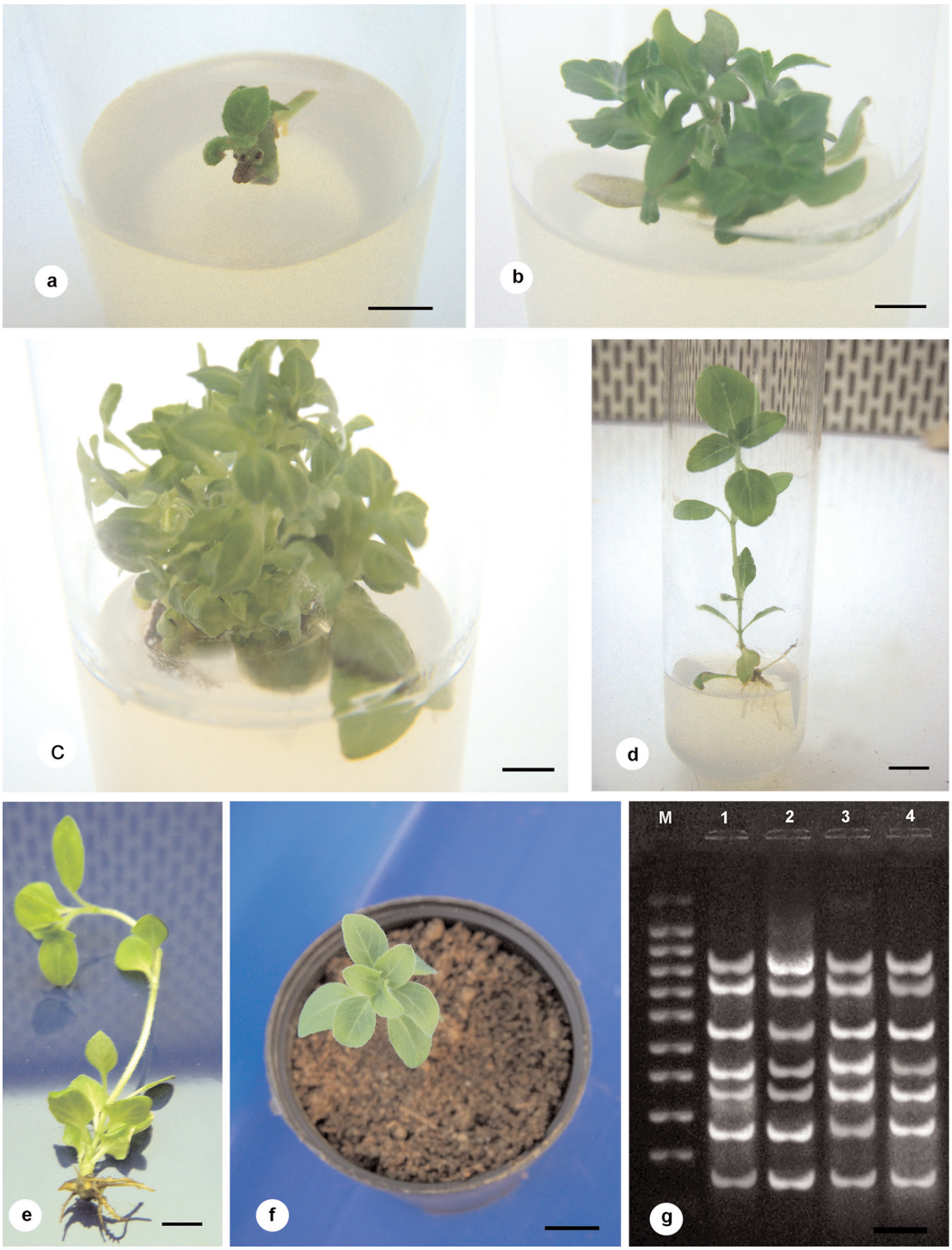

Fig. 1. In vitro clonal propagation of *Ocimum kilimandscharicum* Guerke. (**a**) Shoot proliferation from nodal explant on MS medium supplemented with 1 mg/1 BA after 10–12 days of culture. Bar = 0.5 cm, (**b**) Shoot multiplication on MS medium supplemented with 1 mg/l BA after 4 weeks of culture. Bar = 0.7 cm, (**c**) High rate of shoot multiplication on MS medium supplemented with 1 mg/1 BA. Bar = 0.8 cm, (**d**) Formation of roots from regenerated shoots cultured on ½ MS medium supplemented with 1.5 mg/1 IBA. Bar = 1 cm, (**e**) Well developed root system and complete plant. Bar = 1.0 cm, (**f**) Plantlets developed in vitro, transferred to pot. Bar = 1.6 cm, (**g**) RAPD marker analysis of in-vitro-raised field-grown plants and donor plant of *Ocimum kilimandscharicum*; lane M – λ DNA digested with EcoRI and HindIII as molecular weight marker (100–3000 bp), lane 1 – DNA from mother plant, lanes 2–4 – DNA from randomly selected regenerated plantlets.

TABLE 1. Effect of different concentrations of BA, KN and 2-iP in MS medium on multiple shoot induction from nodal explants of *Ocimum kilimandscharicum*

Growth regulator (mg/l)	Shoot proliferation (%)	Number of shoots/explant	Shoot length (cm)
Control (MS basal medium)	0.00	0.00^{l}	0.00^{g}
MS+BA			
0.5	62.77	1.33 ± 0.06^{ij}	1.28 ± 0.08^{def}
1.0	93.88	6.09 ± 0.05^{a}	3.83 ± 0.11^{a}
1.5	81.66	3.42 ± 0.13^{b}	2.21 ± 0.16^{bc}
2.0	77.77	2.77 ± 0.09^{de}	2.13 ± 0.14^{bc}
3.0	71.66	2.94 ± 0.08^{cd}	1.96 ± 0.12^{bcd}
MS+KN			
0.5	59.44	1.15 ± 0.12^{jk}	1.12 ± 0.02^{ef}
1.0	68.88	2.09 ± 0.11^{gh}	1.58 ± 0.08^{cdef}
1.5	78.33	2.71 ± 0.06^{def}	2.08 ± 0.10^{bc}
2.0	84.44	3.28 ± 0.06^{bc}	2.40 ± 0.13^{b}
3.0	71.66	2.55 ± 0.12^{defg}	1.78 ± 0.04^{bcde}
MS+2-iP			
0.5	53.33	0.83 ± 0.03^{k}	0.88 ± 0.07^{f}
1.0	59.44	1.64 ± 0.09^{hi}	1.08 ± 0.07^{ef}
1.5	72.22	2.28 ± 0.06^{fg}	1.79 ± 0.33^{bcde}
2.0	75.00	2.31 ± 0.14^{efg}	1.84 ± 0.17^{bcd}
3.0	68.33	2.08 ± 0.04^{gh}	1.77 ± 0.16^{bcde}

Values are means ±SE. n = 10–12 (in triplicate). Means followed by same letters do not differ significantly at p = 0.05 by Tukey's HSD test.

ty was maintained up to the fifth subculture on shoot induction medium by regular subculturing. An average 20–30 shoots (shown in Fig. 1c) were obtained from single nodal explants after 18–19 weeks of culture (data not shown). The multiplication rate is higher than reported in other studies (Purohit and Tak, 1992; Hossain et al., 1994; Hiregoudar et al., 2003). After three subcultures the shoot multiplication rate declined. Similar results were recorded in *Gardenia jaminoides* (George et al., 1993) and *Vitex trifolia* (Hiregoudar et al., 2006). The effects of subculture on the multiplication rates achieved in culture are known to differ from one species to another. In *Bacopa monniera*, shoot induction and multiplication increased up to the third subculture passage, after which the frequency and number of shoots decreased (Tiwari et al., 2001). In *Cassia angustifolia*, shoot production increased during subculturing and stabilized in the fifth passage (Siddique and Anis, 2007a). In many plant species, micropropagation requires two media – propagation medium and shoot elongation medium – making the micropropagation procedures cumbersome and uneconomical (Debergh and Maene, 1981). In our present work, shoot multiplication and subsequent elongation were achieved on the same medium. After 18–19 weeks, shoots regenerated in vitro were transferred to rooting medium and rooted successfully after 12–15 days.

ROOTING OF SHOOTS

Elongated shoots (3–5 cm) were excised and placed on half- or full-strength MS medium supplemented with various concentrations of NAA or IBA for induction of roots. No rooting occurred on auxin-free medium. Full-strength MS containing auxins yielded very poor rooting response, but half-strength MS medium supplemented with NAA or IBA and with reduced sucrose concentration (2%) gave us well developed roots within 20–25 days (Tab. 2, Fig. 1d). In many species such as *Lavandula vera* (Andrade et al. 1999), *Chlorophytum borivilianum* (Purohit et al., 1994) and *Dendrocalamus longispathus* (Saxena and Bhojwani, 1993), rooting frequency was higher when shoots were rooted on low-strength MS medium. The rationale behind the favorable effect of reduced macronutrient concentration is that the concentration of nitrogen ions needed for root formation is much lower than for shoot formation and growth (Driver and Suttle, 1987). Of the two types of auxins, IBA was more effective for producing roots than NAA at the different concentrations tested. Shoots formed roots at a high frequen-

TABLE 2. Effect of different concentrations of NAA and IBA in half- and full-strength MS medium on root induction from regenerated shoots of *Ocimum kilimandscharicum*

Growth regulator (mg/l)	Rooting percentage	Number of roots/shoot	Root length (cm)
MS basal medium	0.00	0.00^{g}	0.00^{f}
MS full strength+ PGR			
0.5 NAA	0.00	0.00^{g}	0.00^{f}
1.0 NAA	0.00	0.00^{g}	0.00^{f}
1.5 NAA	0.00	0.00^{g}	0.00^{f}
2.0 NAA	0.00	0.00^{g}	0.00^{f}
0.5 IBA	0.00	0.00g	0.00^{f}
1.0 IBA	21.66	0.54±0.02^{de}	0.22±0.02^{def}
1.5 IBA	25.00	0.82±0.10^{cd}	0.44±0.05^{cd}
2.0 IBA	18.88	0.49±0.05^{e}	0.31±0.01^{de}
MS half strength+PGR			
0.5 NAA	12.22	0.18±0.09^{fg}	0.13±0.07^{ef}
1.0 NAA	0.00	0.00^{g}	0.00^{f}
1.5 NAA	0.00	0.00^{g}	0.00^{f}
2.0 NAA	0.00	0.00^{g}	0.00^{f}
0.5 IBA	15.55	0.41±0.12^{ef}	0.23±0.01^{def}
1.0 IBA	34.44	1.02±0.07^{c}	0.62±0.16^{bc}
1.5 IBA	81.11	3.18±0.06^{a}	1.24±0.09^{a}
2.0 IBA	56.11	1.80±0.11^{b}	0.84±0.07^{b}

Values are means ±SE. n = 10–12 (in triplicate). Means followed by same letters do not differ significantly at p = 0.05 by Tukey's HSD test.

cy (81.11%) on media containing 1.5 mg/l IBA. In this medium the number of roots was highest (3.18±0.06) and their length measured 1.24±0.09 cm. The stimulatory effect of IBA of root formation has been reported in many other medicinal plant species, including *Ocimum basilicum* (Sahoo et al., 1997), *Centella asiatica* (Banerjee et al., 1999), *Murraya koenigii* (Bhuyan et al., 1997) and *Tylophora indica* (Faisal and Anis, 2003). Root development was slow at higher IBA or NAA concentrations; callus formed at the shoot base. These results are in accord with findings from previous studies (Lakshmanan and Dhanalakshmi, 1990; Sahoo et al., 1997).

ACCLIMATIZATION AND FIELD ESTABLISHMENT

Well developed rooted plantlets were gently removed from the test tubes and thoroughly washed with sterile water to remove adhered agar and traces of medium to avoid contamination, and then 53 plantlets were transferred to plastic pots containing soil and vermiculite (1:1) (Fig. 1f). In the first week of transplantation the plantlets were kept covered in a polyethylene tent to provide high humidity and allow sufficient light. The polyethylene cover was removed periodically and progressively whenever leaves appeared to be wet. The polyethylene covers were withdrawn completely after 2–3 weeks of hardening. After 3 weeks the plants were transferred to larger potts filled with soil with organic manure for further growth. Finally the acclimated plants were shifted to field conditions, 81.13% of them having survived. The growth characteristics of plants raised in vitro did not show any significant morphological variations from those of the natural habitat.

RAPD ANALYSIS

Genetic uniformity is one of the most important prerequisites for successful micropropagation of any crop species. A major problem encountered in cells grown in vitro is genetic variation. In many plant species, PCR-based RAPD markers have been used to confirm the clonal fidelity and genetic stability of plants grown in tissue culture and donors (Chawdhury and Vasil, 1993; Rani et al., 1995; Rout et al., 1998; Das and Pal, 2005; Dewir et al., 2005; Chaudhuri et al., 2008; Bhattacharya et al., 2009). We made the DNA fingerprinting profiles of *Ocimum kilimandscharicum* plants regenerated in culture from nodal stem segment explants and the respective donor plants, using 10 RAPD primers.

TABLE 3. Number of amplification products generated with the use of RAPD primers in analyses of genetic fidelity of *Ocimum kilimandscharicum* plants propagated in vitro

Sl. No.	Primer code	Nucleotide sequence (5'-3')	Number of generated bands
1.	MS10C1	GCACGCCGGA	03
2.	MS10C2	AAATCGGAGC	06
3.	MS10C7	CTATCGCCGC	07
4.	MS10C8	CTCTCCGCC	04
5.	MS10C9	GGATGAGACC	03
6.	MS10C10	AGGGCCGTCT	05

Of the 10 primers screened, six primers produced clear and scorable amplified bands ranging from 3 to 7 bands per primer (Tab. 3). Each primer produced a unique set of amplification products ranging in size from 100 to 3000 bp (Fig. 1g with primer MS10C7: CTATCGCCGC). All 6 primers produced a total of 28 bands with an average 4.66 fragments. All 28 scorable bands were monomorphic in nature, indicating homogeneity among the culture regenerates and genetic uniformity with the donor plants. One reason for this may be that multiple shoot bud differentiation took place without an intervening callus phase, reducing vulnerability to genetic changes. The six primers tested in this RAPD study revealed no differences between the mother plants and the plantlets regenerated from nodal stem segments.

In this work we developed the first efficient and reliable micropropagation protocol for in vitro regeneration of *Ocimum kilimandscharicum* from nodal explants. It can be used for large scale propagation and should become a valuable part of strategies for ex situ conservation of this important aromatic and medicinal herb.

ACKNOWLEDGEMENT

We are grateful to the University of Kalyani, Department of Botany, for the use of lab facilities and for other institutional support. Funding for this work came from the University Grant Commission (UGC), New Delhi, an organ of the government of India.

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